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MECHANISMS OF TOXIN PRODUCTION OF FOOD BACTERIA (CLOSTRIDIUM BOTULINUM)

FINAL REPORT

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ABSTRACT

Clostridium botulinum types C and D produced at least three toxins designated as C₁, C₂, and D. When different strains of types C and D were cured of their prophages, they ceased to produce C₁ and D toxins, respectively. Toxin production depended upon the continual participation of specific bacteriophages. Production of C₂ toxin by these same strains, however, was not affected by these bacteriophages. Certain strains of types C and D could be interconverted to either type C or type D by specific bacteriophages. One phage-sensitive strain of C. botulinum type C became a common host to phages of types C and D and also Clostridium novyi type A. When this culture was infected with these phages, it produced C₁ toxin or D toxin or C. novyi toxin, respectively. Further studies showed that bacteriophages governed the production of alpha toxin of C. novyi types A and B. Nontoxigenic derivatives have been isolated from C. botulinum types B (nonproteolytic), but they are not phage-sensitive. Toxigenic Clostridium botulinum and nontoxigenic C. sporogenes, C. subterminale, and C. botulinum-like organisms from a variety of sources have been screened for plasmids. Fifty percent of the 68 C. botulinum isolates carried one or more plasmids ranging in mass from 2.1 to 80 Mdal. A total of 63 plasmids were detected from both nontoxigenic and toxigenic strains. At this time, no phenotypic functions have been assessed for these plasmids and they must therefore be considered cryptic.

INTRODUCTION

Botulism is a neuromuscular disease that is frequently lethal to man and animals. Foodborne, infant, and wound botulism are the clinical forms currently recognized. Foodborne botulism is caused by the ingestion of toxin produced by the bacterium Clostridium botulinum during its growth in feeds and foods. Infant and wound botulism are associated with the growth and toxin production of the organism in the intestines or in the damaged tissue.

Since Van Ermengem's first description of C. botulinum in 1895, additional types have been isolated from different areas of the world. This species now includes a very heterogeneous group of strains that are divided into types A through G on the basis of the antigenic specificity of the neurotoxins that are produced. These different types of C. botulinum can be further divided into four groups according to their biochemical, physiological, and deoxyribonucleic acid homologies. The members of these groups are as follows:

Group 1: Proteolytic types A, B, F, A_F, and B_F.

Group 2: Nonproteolytic types B, E, and F.

Group 3: Nonproteolytic types C and D.

Group 4: Weakly proteolytic type G.

The majority of the human foodborne botulism outbreaks have been caused by types A, B, E, and F. Because of the lethal effect of the neurotoxin, the growth and toxin production by C. botulinum in different food products have been of worldwide concern to food processors and consumers. The incrimination of a given food product in a botulism outbreak can result in loss of consumer confidence of the product and economic disaster to the food industry involved.

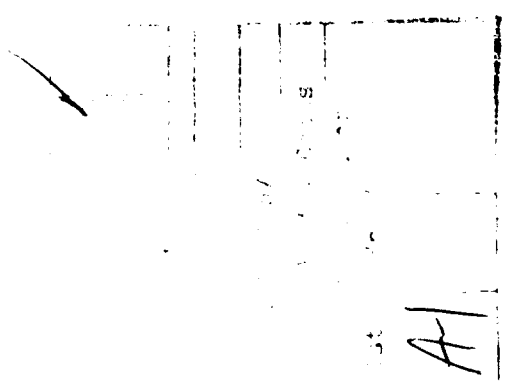

Wound botulism has been caused mainly by proteolytic types A and B, whereas in infant botulism, types A, B, F, and B_F have been involved.

Botulism is also widespread in both domestic and wild birds and animals. Avian botulism outbreaks (type C and occasionally type E) involve thousands of wild birds and domestic birds such as broiler chickens and turkeys. Animal botulism (caused by types C and D) also occurs in different areas of the world causing serious loss of animal life in Africa.

During the course of this study, we discovered botulism type E as the cause of a mysterious disease that has plagued the pond-reared fish industry in the United States for over 25 years (see Appendix). Since 1979, over 2.5 million juvenile salmonids have died from botulism in Washington and Oregon States. This disease has resulted in millions of dollars loss to the industry each year.

Type G was first isolated in Argentina from a soil sample and, more recently, it has been isolated at autopsies from humans who have died from unknown causes in Switzerland.

The most important characteristic in the identification and differentiation of these C. botulinum types is the production of lethal neurotoxins. With certain types and strains of C. botulinum, this characteristic is occasionally lost during transfer in laboratory media. In addition, nontoxigenic clostridia resembling C. botulinum have frequently been isolated from aquatic and terrestrial environments. These observations of nontoxic clostridia which physiologically and biochemically resemble C. botulinum have been the basis for investigating the mechanism that governs the toxigenic characteristic.



SUMMARY OF RESULTS

Relationship of Bacteriophage to Toxigenicity of *C. botulinum* Types C and D

Initially *C. botulinum* types C and D (group 3) were studied because the strains of these types were known to lose toxigenicity when transferred in laboratory media. *C. botulinum* types C and D produce at least three different toxins designated as C_1 , C_2 , and D.

The predominant toxins produced by type C and D strains are designated as C_1 and D, respectively. Most strains of type C and a few strains of type D also produce low titers of another toxin designated as C_2 . This toxin, however, was detectable only after activation with trypsin.

Our studies have shown that type C and D strains ceased to produce the dominant C_1 and D toxins when they were cured of their TOX^+ bacteriophages. The production of the C_2 toxin, however, was not affected. Reinfection of these nontoxic derivatives with specific TOX^+ bacteriophages again induced production of C_1 and D toxins.

Subsequent studies have shown that one strain of type C ceased to produce both C_1 and C_2 toxin when it was cured of its TOX^+ bacteriophage. This nontoxic derivative was induced to produce the C_1 toxin but not the C_2 toxin by re-infection with the TOX^+ phage from the toxigenic parent. This is the first time that a $TOX C_2^-$ derivative has been isolated from a $TOX C_2^+$ culture. The mechanism governing the production of the trypsin-activated C_2 toxin is not known.

When certain type C and D strains were cured of their bacteriophages, they ceased to produce their dominant C_1 and D toxins and became sensitive to both the bacteriophages of both type C and D. The type C phages induced the production of C_1 and the type D phages induced D toxin. These

cultures therefore were interconverted to either types C or type D merely by exchanging the TOX⁺ phage.

Interspecies Conversion of C. botulinum Type C to C. novyi Type A by Bacteriophage

Both C. botulinum and C. novyi produce lethal toxins. The C. botulinum organisms produce neuroparalytic toxins that are responsible for botulism in man and animals. The C. novyi species produce several different toxins and are often involved in gas gangrene infection of man and other diseases in animals. C. novyi is divided into A, B, C, and D on the basis of the production of toxins and other active agents. Strains of type A produce the lethal alpha and gamma toxins, but otherwise are closely related to C. botulinum types C and D.

When a C. botulinum type C strain was cured of its bacteriophage, it not only ceased to produce the C₁ toxin, but it became a common host to the phages of C. botulinum type C and type D and also C. novyi type A. Infection of this nontoxigenic derivative with type D^{TOX⁺} phages converted it to a C. botulinum type D. If this strain was cured of the type D^{TOX⁺} and reinfected with C. novyi type A phage TOX⁺, then it produced the lethal alpha toxin and became indistinguishable from other strains of C. novyi type A. Phage-sensitive derivatives of this culture could be reinfected with type C phage and the culture was induced to again produce C₁ toxin. A phage-sensitive strain of C. botulinum type C therefore could be converted to C. botulinum type C or type D or to C. novyi type A by interchanging the bacteriophages. The most important characteristic in the identification and differentiations of C. botulinum type

C and D and C. novyi type A therefore is the specific bacteriophages that they carry and the toxins produced.

Relationship of Bacteriophage to Toxigenicity of C. novyi

The observation that specific bacteriophages from C. novyi type A could induce nontoxigenic derivatives of C. botulinum type C to produce C. novyi alpha toxin suggested that the phages of C. novyi probably govern the alpha toxin production in C. novyi types A and B. In subsequent studies, the production of alpha toxin was shown to depend upon the continued participation of specific phages. Other toxins and biologically active compounds however were not affected by the phages.

Relationship of Bacteriophage to Toxigenicity of Proteolytic Strains of C. botulinum Types A and F

This group of organisms are not only different from types C and D in biochemical and physiological characteristics, but also in the phage and host relationship. With types C and D, this relationship is pseudolyso-genic, and phage-sensitive derivatives can routinely be isolated from the toxigenic parent culture. In contrast with the proteolytic strains of C. botulinum, this phage-host relationship is true lysogeny. It is therefore very difficult to cure proteolytic strains of their bacteriophages. A strain of type A has been cured of four bacteriophages following treatments with mitomycin C-acridine orange or acriflavine. This phage-sensitive derivative, however, continues to produce toxin. Following the curing of the fourth phage, antiserum was prepared against the toxin to determine whether different toxins might be produced by type A in a system analogous to C. botulinum types C and D. This antiserum, however, neutralized the

the undiluted toxin of the parent strain carrying the four phages. This indicates the same toxin or toxins were produced by both the parent and derivative cured of phages.

In similar experiments, a strain of proteolytic type F has been cured of two bacteriophages and it also continues to produce type F toxin. Based upon these data, the relationship of phages to toxigenicity in types C and D differs from those of proteolytic strains.

Clostridium sporogenes and proteolytic strains of C. botulinum types A, B, and F are biochemically and physiologically closely related. The main characteristic that differentiates the two species is the production of neurotoxins by C. botulinum strains.

Since C. sporogenes could be a nontoxigenic variant of the proteolytic types of C. botulinum, they were tested for sensitivity to phages of 36 strains of types A, B, F, and Af. None of the C. sporogenes were sensitive to the C. botulinum phages.

Because of the possibility that C. sporogenes might carry phages that are antigenically related to C. botulinum and are therefore immune to infection, several strains of C. sporogenes were cured of their prophages. Of the four strains tested, three have been cured of at least one of their prophages following mitomycin C-acridine orange treatment. Lysates of 79 strains of C. botulinum were tested on these phage sensitive derivatives. Some culture supernatants of C. botulinum type B produced lytic or inhibitory reactions on C. sporogenes lawns, but none of these reactions were caused by bacteriophages. Filtrates from C. botulinum strains were also cultured with the C. sporogenes derivatives, but toxicity was not induced.

In most relationships between phage and toxigenicity, the continued participation of the phage is necessary to maintain toxicity. With Staphylococcus aureus, this relationship is reversed and the cultures ceased to produce toxin when they are infected with specific bacteriophages. When this species was cured of their phages, they again produced toxins. C. sporogenes strains were therefore assayed for toxin before and after they were cured of their prophages to determine whether this phenomenon would also occur with these clostridia. All cultures, however, remained non-toxigenic during 3 days of incubation at 30°C in cooked meat medium.

Relationship of Bacteriophages to Toxigenicity of Nonproteolytic C. botulinum Types B, E, and F.

The phage-host relationship of this group of organisms is true lysogeny and closely resembles the proteolytic strains of types A, B, and F.

Toxigenic strains of nonproteolytic types B, E and F have been treated with mitomycin C and cultured in media containing acridine orange or acriflavine and isolated cultures tested for phage sensitivity and toxin production.

With type B, three groups of derivatives were obtained. The first group ceased to produce toxin, but were not sensitive to the phages of the toxigenic parent culture. The high frequency of isolating these nontoxigenic derivatives from two different toxigenic type B strains indicates that unidentified phages or possibly plasmids could be controlling toxigenicity. These nontoxigenic derivatives have been induced to lyse by treatment with mitomycin C and tailless phage heads have been observed in electronmicrographs. These nontoxigenic strains of type B were therefore cultured in broth containing sediments from which other toxigenic type B

organisms have been isolated. Numerous phages infecting the nontoxigenic type B cultures were isolated, but they did not induce toxin production.

The second group of type B derivatives isolated have been cured of one of their two phages, but they continue to produce toxin. In the third group, one isolate continued to produce toxin, but typsin treatment was required to activate the toxin. This isolate was not phage-sensitive.

Nontoxigenic derivatives have also been isolated from a strain of type E. This derivative is not phage-sensitive and both the toxigenic and nontoxigenic strains carry the same phages.

A phage-sensitive derivative has also been isolated from nonproteolytic type F. This strain, however, continues to produce toxin.

These data indicate that bacteriophages do not appear to play the same role in toxigenesis of groups 1 and 2 as they do in type C and D strains in group 3.

Several explanations are offered for the inability to convert nontoxigenic derivatives of nonproteolytic types B and E to the toxigenic state: (a) incorrect methodology is being used to detect the phages; (b) bacteriophages are defective; (c) nontoxigenic derivatives are mutants that are resistant to parent cultures; or (d) plasmids mediate the toxigenic characteristic.

Plasmids of *C. botulinum*

A great deal of effort has been devoted to the development of methodology for lysing the different *C. botulinum* and closely related species and the subsequent plasmid isolation and purification. In many of the preparations, it was difficult to extract plasmids without extensive single or double stranded degraded DNA contamination. This resulted in

dense fluorescence along the entire lane on agarose gels, making it difficult to visualize plasmids bands. In most strains, this problem has been corrected and plasmids ranging in molecular weight from 2.1 to 80 mdal (megadaltons or 1×10^6 daltons) were found in 44 (or 53%) of the 83 strains.

No phenotype has been ascribed to any of the 67 plasmids isolated from 44 strains of C. botulinum and related nontoxigenic species. However, all of the proteolytic F strains tested were found to carry a single 14.5 Mdal plasmid and all of the type G strains harbored a single 80 Mdal plasmid. In addition, four of the six nonproteolytic F strains contained a single 2.2 Mdal plasmid. Despite their widely different geographical origins, these were the only plasmids detected in these strains. It is possible these plasmids may code for some common product among the strains of each type such as botulinum toxin. It is uncertain whether plasmids are involved in the toxigenicity of the remaining C. botulinum types since many strains within a group did not carry plasmids, and both similar and different profiles were observed in the plasmid-carrying strains.

The plasmids found may code for functions important for survival of the host organism at the original site of isolation. For example, plasmids may be responsible for colonization or adherence factors enabling some proteolytic group 1 strains to establish infections in sites where they are not normally found, such as in wounds or infant intestinal tracts. Another example may be the production of bacteriocins. Nonproteolytic B strain 17844 carries a 2.1 Mrad plasmid and also produces a lytic factor for other type B strains (unpublished results). Similarly, the three multiple plasmid-carrying nontoxigenic group 2 strains also produce bacteriocins active against toxigenic group 2 cultures.

This is the first demonstration that plasmids are widespread in C. botulinum and related species. Work is continuing in this laboratory to determine their role in the growth of the host organisms. Whatever their function, they may prove valuable in future genetic studies of C. botulinum.

LIST OF PUBLICATIONS

Published

1. Relationship of Bacteriophages to the Toxigenicity of Clostridium botulinum and Closely Related Organisms
2. Botulism in Juvenile Coho Salmon (Oncorhynchus kisutch) in the United States

To be Published

1. Toxins Produced by a Nontoxigenic Derivative of Clostridium botulinum Type C Infected with Different Bacteriophages
2. Further Observations of Botulism in Salmonids in the United States
3. Subtypes of Clostridium botulinum Types C and D and their Relationships to Bacteriophages
4. Plasmids in Clostridium botulinum and Related Clostridium Species

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LIST OF APPENDICES

1. Relationship of bacteriophages to the toxigenicity of *Clostridium botulinum* and closely related organisms
2. Botulism in juvenile coho salmon (Oncorhynchus kisutch) in the United States

BOTULISM IN JUVENILE COHO SALMON (*ONCORHYNCHUS KISUTCH*) IN THE UNITED STATES

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ABSTRACT

Eklund, M.W., Peterson, M.E., Poysky, F.T., Peck, L.W. and Conrad, J.F., 1982. Botulism in juvenile coho salmon (*Oncorhynchus kisutch*) in the United States. *Aquaculture*, 27: 1-11.

Botulism type E was first recognized as a major cause of fish mortality in the United States in 1979. This disease caused an estimated loss of 1¼ million juvenile salmon reared in earth-bottom ponds during the summer and fall of 1979 and 1980. The botulism outbreaks, preliminary laboratory results of experimental botulism, and precautions to be taken by fish farm employees are discussed.

INTRODUCTION

Botulism is a neuromuscular disease that is frequently lethal to man and animals. Foodborne, infant, and wound botulism are the three clinical forms that are currently recognized. Foodborne botulism is caused by the ingestion of toxin produced by the bacteria *Clostridium botulinum* during its growth in feeds and foods. Infant and wound botulism are associated with the growth and toxin production of the organism in the intestines or in damaged tissue.

Based upon the production of antigenically specific neurotoxins, the species *C. botulinum* is divided into different types designated by the letters A through G. The distribution of these toxin types varies with the geographical area, but in general type E is the most prevalent in marine and freshwater environments of the Northern Hemisphere. All of the toxins produced by the different types are lethal, but the sensitivity of man and different animal species varies with the *C. botulinum* toxin type.

Botulism or "bankrupt disease" was first reported in pond-reared trout in Denmark by Huss and Eskildsen in 1974. The source of the toxin in these

outbreaks was from the growth of *C. botulinum* type E in feeds stored at nonrefrigerated temperatures and from cannibalism of dead fish accumulating in the bottom of the rearing ponds.

This report describes the first recognized type E botulism outbreak in pond-reared fish in the United States. These outbreaks occurred during the fall of 1979 and 1980 and resulted in the loss of over 1¼ million juvenile coho salmon (*Oncorhynchus kisutch*) in Washington and Oregon State Hatcheries. The diagnosis of the disease, multiplication of *C. botulinum* type E in pond sediments, preliminary laboratory experiments of botulism in juvenile salmonids, and precautions to be taken by hatchery employees are also discussed.

MATERIALS AND METHODS

Botulinum toxin assay and neutralization tests

The flesh or intestines of fish dying with botulism symptoms were ground and extracted with 2 ml of physiological saline or gelatin-phosphate buffer (Duff et al., 1956). After extracting for 1 h, the samples were centrifuged and 0.5 ml of supernatant fluid or fluid treated with trypsin (Duff et al., 1956) was injected intraperitoneally (I.P.) into Swiss Webster mice weighing 18 to 26 g. Toxin titrations were made by diluting samples in log intervals in gelatin-phosphate buffer and injecting mice I.P. with 0.5 ml of each dilution. Sediment samples from the rearing ponds were centrifuged and supernatant fluids were assayed for toxin before and after trypsin treatment. Water from the hatchery ponds was concentrated by dialyzing against polyethylene glycol (Kahn, 1959) and assayed for toxin. Toxin neutralization tests were made using the mouse protection test and monovalent *C. botulinum* antiserum as outlined by Eklund and Poysky (1972).

Isolation and enumeration of C. botulinum type E from fish and sediments

TPG medium (5% trypticase, 0.5% peptone, and 0.4% glucose) containing a final concentration of 0.1% sodium thioglycollate was used for culturing type E organisms. Aliquots of fish intestines, sediments, or their enrichment cultures were streaked onto egg yolk agar plates. Following incubation, typical isolated colonies displaying iridescence were selected and picked into TPG broth and tested for toxicity after 3 days of incubation at 30°C. Type E organisms were enumerated in sediment samples using the TPG medium and the three-tube Most Probable Number procedure. The presence of *C. botulinum* type E toxin was used as confirmation of type E growth.

Sensitivity of fish to C. botulinum type E toxin

Type E toxin was produced in TPG medium at 30°C using isolates from

the hatchery sediments or the toxin was extracted from the flesh or intestines of fish displaying botulism symptoms. Fish weighing 8 to 12 g were tested for their sensitivity to untreated and trypsin-treated type E toxin by the intraperitoneal and oral routes.

RESULTS AND DISCUSSION

Mortality in salmon hatcheries and confirmation of botulism

This report discusses two botulism outbreaks at the Washington State Elokomin Hatchery in 1979 and 1980 and one at the Oregon State Klaskanine Hatchery in 1980.

Outbreak at Elokomin in 1979

Pond 23 is a 0.53-acre earth-bottom pond with an average water depth of 3.5 ft and a maximum depth of 5 ft near the outlet. It has an inflow of water of 17 ft³/s. In May 1979, Pond 23 was cleaned and repopulated with approximately two million coho salmon weighing 1 to 2 g each.

On 28 August, the fish began to be hypersensitive and nervous, and within a 3-day period, losses increased from 20 to 800 fish per day and remained at this level for approximately 1 week (Fig. 1). The mortality rate then began to double each day until the losses reached 70 000 fish per day. On 21 September, the majority of the survivors were moved to another earth pond designated Number 22. The fish remaining in Pond 23 continued to get botulism and within a 3-week period there were no survivors. The mortality rate in Pond 22 decreased to 2000 fish per day for a 2-week period and then suddenly increased again to 22 000 fish (Fig. 1). The daily losses remained in the range of 16 000 to 19 000 for an additional 4 days; then decreased to 2000 fish per day and continued at this level until the first part of November when the outbreak stopped. This outbreak resulted in a total loss of approximately one million fish.

Fish with botulism appeared to lose their equilibrium and would swim on one side and then on the other. They were unable to swim against the water current and, as a result, were forced to the outlet screen or to low flow areas where they would lie on their sides. When disturbed, they would move toward the surface in a jerking motion only to sink again to the bottom as though they were tail-heavy. Once the fish developed symptoms, death was inevitable. The symptoms persisted for many hours in some fish, whereas in others death occurred rapidly. The water temperature of the ponds ranged from 14 to 16.6°C during the months of July through the middle of October while the outbreak was in progress. During the latter part of October when the botulism outbreak stopped, the water temperatures decreased to temperatures in the range of 10.5 to 14.5°C.

The cause of the outbreak was determined during the middle of October

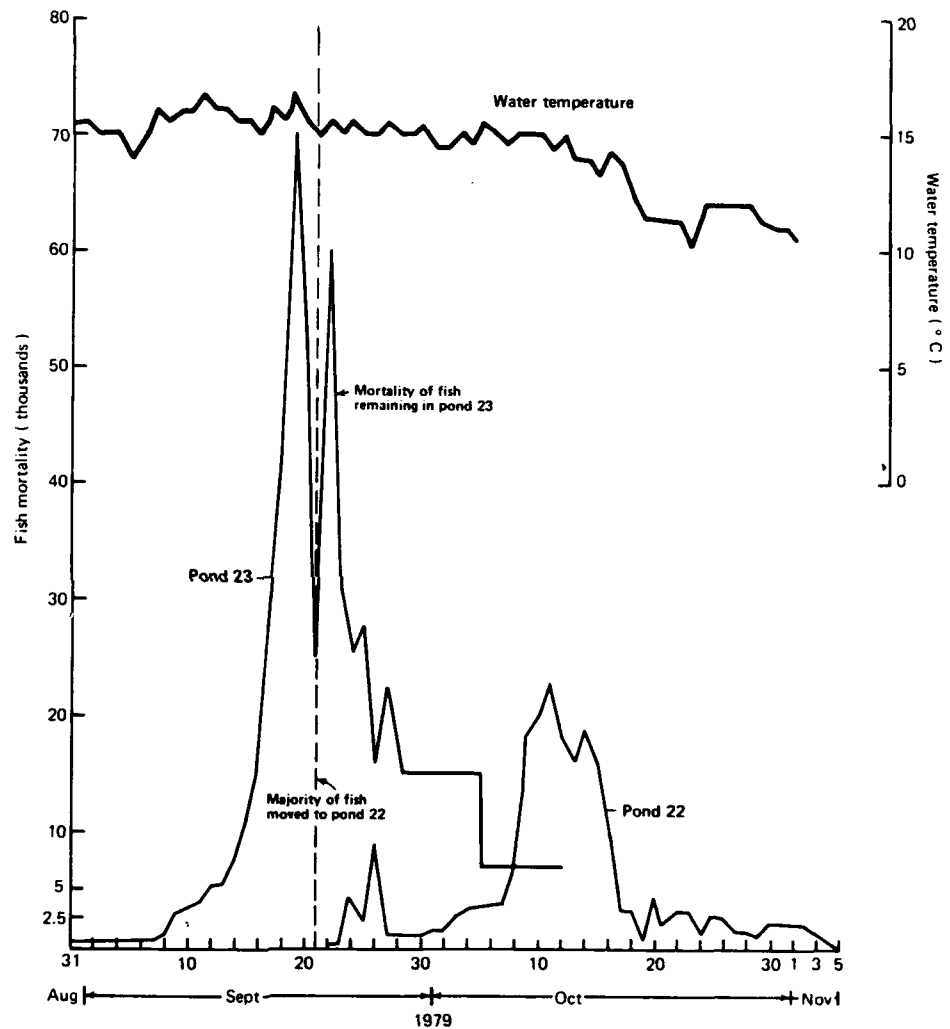


Fig. 1. Fish mortality from botulism outbreak at Elokomin Hatchery in 1979. After 28 September, the fish mortality in Pond 23 is reported as the daily average per week.

when *C. botulinum* type E toxin was demonstrated in the stomach and intestinal contents and flesh of morbid fish. The last 10–15 mm of the intestinal tract contained very viscous yellow-orange fecal material. This suggested a metabolic disorder of the digestive system or a state of constipation, a symptom frequently observed in animal or human botulism. No other pathogenic conditions were observed.

When the intestines of the morbid fish were ground and extracted with gelatin-phosphate buffer, many of the supernatant fluids of the extracts

produced characteristic botulism symptoms in mice and in juvenile salmon when the toxin was introduced by the I.P. route. When toxic extracts were mixed with *C. botulinum* type E antitoxin, they were no longer lethal to either mice or fish. Antitoxins from other *C. botulinum* types, however, did not offer this same protection. This specificity of neutralization confirmed the presence of *C. botulinum* type E toxin in the fish intestines and flesh.

Typical results of the toxin assays from morbid fish are summarized in Table I. The titers of the intestinal contents ranged from 2 to 200 MLD per

TABLE I

Detection of type E toxin in intestines of fish with botulism symptoms

Date collected	No. toxic/no. tested	Range of toxin titers ^a
5 October	2/9	2-200
9 October	5/5	2-200
9 October	2/10	2-100
16 October	16/20	2-200
18 October	3/6	2-20

^aFish containing 100 MLD of toxin per ml of intestinal extract frequently contained 20 MLD of toxin per g of flesh.

ml of extract or a total of 6 to 600 MLD per fish stomach and intestines. When fish intestines contained greater than 100 MLD toxin per ml, 20 MLD of toxin was often found in the flesh. In some cases, toxin was detectable in the flesh but not detectable in the intestines. Trypsin treatment increased the toxin titers of some intestinal samples, but did not increase the titers of the extracts from the fish flesh.

The bacterium *C. botulinum* type E was isolated numerous times from the intestines of the fish or from pond sediments which contained 75 000 type E organisms per g. The toxin from the type E culture filtrates was lethal to fish by the oral and intraperitoneal routes and produced botulism symptoms identical to those observed in the botulism outbreaks at the hatcheries. Type E antitoxin again protected the fish from the lethal toxin.

Outbreak at Elokomin Hatchery in 1980

Following the 1979 botulism outbreak, the sediments of Pond 23 were removed and the pond was relined with new gravel. The pond was repopulated with one million coho salmon weighing 1 to 2 g each in December 1979. Starting in March, sediment samples from Pond 23 were collected and *C. botulinum* type E populations determined. The first samples contained 400 type E organisms per g (Table II). The population increased to 24 000 by May and then remained relatively constant for the next 2 months. In

TABLE II

Growth of *C. botulinum* type E in sediments of Pond 23 in 1980

Month	Water temperature (°C)		Type E organisms per g sediment
	Range	Mean	
March	5.0—8.3	7.2	400
April	6.1—12.8	9.4	1500
May	7.2—10.6	10.6	24000
July	11.1—20.0	15.6	15000
August	6.1—18.3	15.6	15000
15 September	5.5—15.6	13.3	46000
24 September	10.6—13.3	12.8	240000
October	6.1—14.4	10.6	150000

September, the number of type E organisms increased to 46 000 and continued to increase until some of the sediment samples contained 240 000 per g. The highest population of type E occurred within 20 ft of the outlet screen where waste feed, fecal material, dead fish, and other sediments accumulated to depths of 4 to 6 inches. Lower populations of type E (110 to 4600 per g) were present in other areas of the pond where the accumulation of sediments was 1 to 2 inches deep. The water temperatures of the pond started at 5.0 to 8.3°C in March, increased to a high of 20°C in July, and then decreased thereafter. Increases in type E populations did not appear to be correlated with the water temperatures in the pond.

The fish mortality during the period of December 1979 to September 1980 remained at less than 20 fish per day. The same mortality pattern observed in 1979, however, began to reappear during the first 2 weeks in September 1980. Fig. 2 shows the daily losses increasing from 20 to 400 per

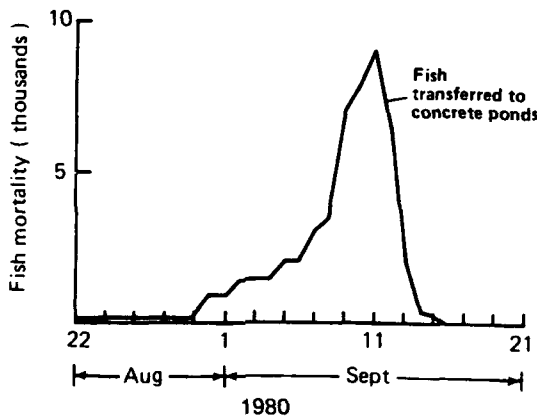


Fig. 2. Fish mortality from botulism outbreak at Elokomin Hatchery in 1980.

day during the latter part of August and then doubling each day until the losses reached 9000 fish. Based upon the increases in type E populations in the sediments and the previous year's experience with botulism, the fish were transferred to concrete ponds and the mortality began to decrease immediately. Within 5 days, the fish losses were less than 100 per day. No further problems were encountered with this fish population.

Klaskanine Hatchery outbreak in 1980

The earth bottom pond referred to as the Lake at the Klaskanine Hatchery is of comparable size and has about the same water flow as Pond 23 at the Elokomini Hatchery. The Lake has had epizootics of unknown etiology since 1960. The causative agent of the outbreaks was not known until 1980 when we demonstrated type E toxin in the digestive tract and flesh of morbid fish. This outbreak resulted in losses of 260 000 yearling coho salmon out of an original population of 871 000 fish.

The mortality pattern was similar to that observed at the Elokomini Hatchery with the exception that the outbreak was somewhat self-limiting. The losses started to increase on 14 September from 20 to over 200 fish per day. For 2 weeks, the mortality remained at 800 fish per day, and then continued to increase until the daily loss was 24 000 fish. After that, the losses steadily decreased and within 2 more weeks, the outbreak stopped (Fig. 3). The Lake sediments contained smaller populations of type E, 2400 to 11 000 organisms per g, than the Elokomini Pond 23. In addition, Lake sediments contained other bacteria which inactivated type E toxin when sediments were cultured in TPG medium. When sediments from the Elokomini pond were cultured, this inactivation of toxin was not observed. These microbial differences could have contributed to the magnitude and duration of the outbreaks at the two hatcheries. The water temperature of the Lake ranged from 12 to 15.5°C during the month of September and the early part of October and decreased during the latter phases of the outbreak.

PRELIMINARY LABORATORY EXPERIMENTS AND SOURCE OF TOXIN

When fish were force fed capsules containing 0.1 ml of different titers of filter-sterilized type E toxin, the minimum lethal dose for a 10-g fish held in 15°C water was 200 MLD (based upon trypsinized mouse intraperitoneal toxin titers). Immediately following the development of botulism symptoms and death, the digestive tracts of these fish were extracted and assayed for unabsorbed toxin. A toxin titer of 400 MLD was detected in the stomach and intestines of the fish if they had been fed 2000 MLD of toxin. In comparison, fish dying of botulism in the hatcheries often contained 6 to 600 MLD of type E toxin in their digestive tracts. Some of these hatchery fish therefore were exposed either to high concentrations of toxin, or to a continuous source of toxin, or the type E organism grew and produced toxin in the in-

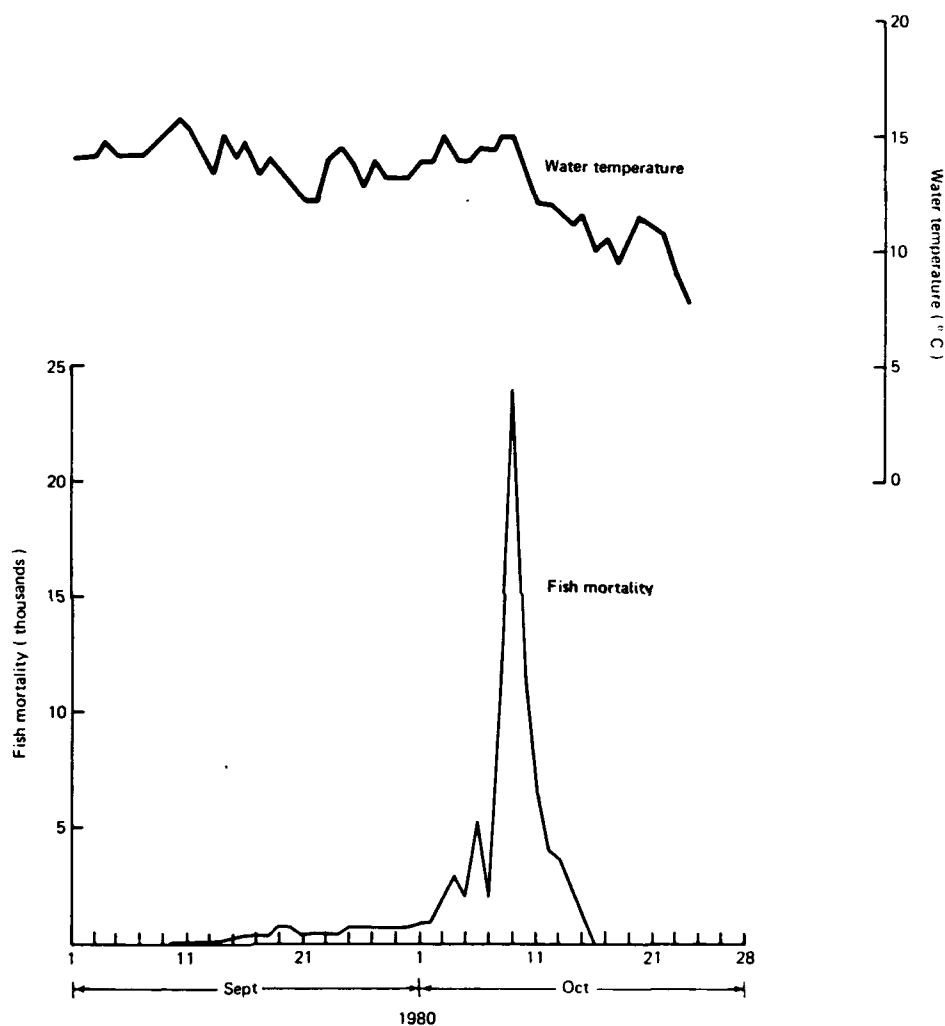


Fig. 3. Fish mortality from botulism outbreak at Klaskanine Hatchery in 1980.

testines of some of the fish in a manner analogous to the development of infant botulism in humans. The fish feed and different samples collected from the hatchery ponds were therefore assayed to determine the source of the type E toxin. Oregon moist pellets were used by both hatcheries and were stored in freezers until they were used. Neither toxin nor viable *C. botulinum* organisms were detected in these pellets. Water samples were collected at different times, but toxin was not detectable even after they had been concentrated 60 times by dialyzing against polyethylene glycol. Toxin was not present in detectable concentrations in any of the 93 pond sediment samples collected at the Elokomin Hatchery, but 2 MLD of type E toxin

was demonstrated in one of the 16 samples collected at the Klaskanine Hatchery. Large numbers of tubifex worms were frequently present in the sediments from both hatcheries, but only one of the 28 tubifex samples contained type E toxin (2 MLD per g) which was detectable only after trypsin activation.

Dead fish accumulating on the pond bottoms were the greatest source of toxin often containing 200 MLD of toxin per g. This undoubtedly was the combined effect of the toxin consumed by the fish prior to death and the subsequent growth and toxin production by *C. botulinum* type E in the dead fish. Cannibalism was not evident in the fish that had been dead for 1 or 2 days, but could have occurred in fish that had been dead for longer periods of time and where advanced decomposition obscured signs of cannibalism.

We concur with Huss and Eskildsen's (1974) recommendations that dead fish undergoing decomposition are a potential source of toxin and therefore should not be permitted to accumulate in the pond bottoms. Because of the depths and cloudiness of the water in some ponds, this practice, however, often cannot be followed. Controlled experiments therefore are currently in progress to determine a method for controlling the growth of type E in the sediments, which would also eliminate sources of low titered toxins in the sediments and tubifex worms.

PRECAUTIONS FOR FISH FARM EMPLOYEES

C. botulinum bacteria produce the most potent toxin known. Because of the dangers of this toxin to man and animals, extreme precautions should be taken by all fish farm and hatchery personnel. The dead fish should be incinerated or placed in trenches away from domestic water supplies and buried under a layer of quick lime and soil. Otherwise, the fish botulism outbreaks could be extended to domesticated or wild bird and animal populations.

Employees should be informed that the *C. botulinum* organism and its toxin are potentially dangerous to themselves and their families. Great care therefore should be exercised when employees work with dead fish as the feces and flesh will often contain toxin which could be absorbed through cuts or even taken internally if improper hygiene were followed.

Type E bacteria could potentially grow and produce wound botulism (Dezfulian and Dowell, 1980) if it is introduced into damaged tissue of humans. In addition, children under 1½ years of age also can develop infant botulism by the growth of the *C. botulinum* bacteria in the intestines. *C. botulinum* types A, B, and F are the only types that have caused infant botulism since it was first discovered in 1975 (Arnon et al., 1977), but the potential of type E infant botulism exists.

C. botulinum type E has several characteristics which increase its dangers to humans in the form of food poisoning. It can grow and produce toxin at temperatures as low as 3.3°C and, because of its nonproteolytic characteris-

tics, its growth in foods cannot be detected by off-odors and off-flavors. If type E is brought into the household, especially in high numbers, through contaminated clothing or improperly washed hands, it could be introduced into cooked or uncooked meats, fish, and vegetables. Toxin can be produced in these contaminated foods within 24 h at room temperature, 1 week at 10° C, and 3 weeks at 3.3° C. Consumption of these foods therefore could result in fatal forms of botulism-food poisoning. It is important that good sanitation and food handling practices be used in the home at all times. Perishable foods should be stored below 3.3° C and consumed within a few days. For longer storage, the foods should be frozen. Freezing does not destroy the organism or its toxin, but it will stop *C. botulinum* from growing and producing toxin. If canning, pickling, smoking, drying, or other methods are used to preserve foods in the home, approved recommended preservation procedures should be followed.

It is therefore essential that employees wear protective clothing and gloves whenever working with the diseased fish and that good personal hygiene be followed. Antiserum against the *C. botulinum* toxins is available for humans, but its effectiveness in controlling botulism depends upon the advancement of the disease. Because of the extreme toxicity of botulinal toxins, prevention of the disease is recommended.

The sediments of the ponds in which botulism has occurred contain unusually large numbers of *C. botulinum* type E organisms. These sediments should not be used as fertilizer for home vegetable or flower gardens or for other purposes. Instead the sediments should be buried in the same manner as recommended for dead fish.

Botulism has only been demonstrated in small fish from hatcheries. If botulism is demonstrated in fish of marketable size, it is very important that during the outbreak none of the fish, healthy or morbid, be consumed by man or animals as botulism symptoms could be advancing in the fish at different rates, and the flesh could contain lethal levels of toxin.

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RELATIONSHIP OF BACTERIOPHAGES
TO THE TOXIGENICITY OF *CLOSTRIDIUM BOTULINUM*
AND CLOSELY RELATED ORGANISMS

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INTRODUCTION

The most important characteristic in the identification and differentiation of the pathogenic clostridia is the production of toxins. Based upon the production of antigenically specific neurotoxins, the species *Clostridium botulinum* is divided into types A through G. Even though the different toxin types represent a heterogenous group of strains, they have been placed into one species because of the similar pharmacological action of the toxins. When biochemical, physiological, and serological characteristics and deoxyribonucleic acid homologies are used to characterize the different *C. botulinum* strains, this species can be separated into four groups. Group I cultures are proteolytic and produce toxin types A, A_F, B, and F; group II cultures are nonproteolytic and produce toxin types B, E, and F; group III cultures are nonproteolytic and produce toxin types C₁, C₂, and D; and group IV cultures are weakly proteolytic and produce toxin type G.

The loss of the toxigenic characteristic has been observed in pure cultures of *C. botulinum* during culture in laboratory media. In addition, nontoxigenic clostridia resembling *C. botulinum* have been isolated frequently from aquatic and terrestrial environments. The occurrence of nontoxigenic

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cultures coupled with the observations that all types of *C. botulinum* carry bacteriophages (2,3,14,24) suggested that the production of toxins by *C. botulinum* might be mediated by bacteriophages or plasmids analogous to the production of toxin by *Corynebacterium diphtheriae* (1,10,11).

This report provides evidence for the involvement of specific bacteriophages in the toxigenicity of *C. botulinum* types C and D and closely related organisms.

BACTERIOPHAGES AND THE TOXIGENICITY OF *C. BOTULINUM* TYPES C AND D

C. botulinum types C and D produce at least three different toxins designated as C₁, C₂, and D (5,7,17). Type C strains produce predominantly C₁ toxin and minor amounts of C₂ and D toxins. In contrast, type D strains produce predominantly D toxin and minor amounts of C₁ and C₂ toxins. The minor toxins are not produced by all strains of types C and D.

The relationship of bacteriophages to the toxigenicity of *C. botulinum* was first observed in type C and D strains (4,6,15,16). Nontoxigenic derivatives were isolated from toxigenic strains following acridine orange or ultraviolet irradiation treatments. When these nontoxigenic derivatives were infected with bacteriophages from the toxigenic parent culture, toxigenic isolates were again recovered.

The roles that different bacteriophage play in the toxigenicity and in the interrelationship of *C. botulinum* types C and D and closely related organisms are discussed in further detail in the following sections of this paper.

Toxigenicity of Type C Strains

A bacterial culture is generally immune to the infection by bacteriophages that it carries or to antigenically related bacteriophages that are produced by other cultures. In order to determine the relationship of bacteriophages to the toxigenicity of a bacterial strain, one must therefore isolate bacteriophage-sensitive derivatives, preferably from known toxigenic strains.

Strain 468C was the first culture used in our laboratory to study the involvement of bacteriophages in the toxigenicity of type C cultures. This strain was grown in trypticase, yeast-extract glucose (TYG) medium containing acridine orange (AO) or cultures in logarithmic phase of growth were treated with ultraviolet (UV) irradiation to cure them of their prophages. Surviving colonies that developed on TYG agar

following anaerobic incubation were tested for sensitivity to the parent phages. After a 60-second treatment with ultraviolet light, 15 of 106 cultures tested were cured of prophages and concomitantly ceased to produce C_1 and D toxins. In comparison, 2 of 68 colonies tested from the acridine orange treatment were cured of their prophages and toxigenic characteristic.

The inability of these phage-sensitive cultures to produce C_1 and D toxins was confirmed during subsequent passages in laboratory medium and indicated that the loss of the toxigenic characteristic was permanent. Later, it was learned (5) that the production of C_2 toxin was not governed by bacteriophages and that phage-sensitive cultures continued to produce C_2 toxin. This toxin, however, was detectable only after activation with trypsin. For simplicity, the term "nontoxigenic" will be used hereafter in reference to cultures that failed to produce C_1 and D toxins.

To determine whether more than one bacteriophage was produced by 468C, each phage-sensitive "nontoxigenic" derivative was tested for its sensitivity to the lysates of other cured derivatives using the agar-layer procedure (4,6). Derivative AO28 was the only isolate that was sensitive to the lysates of other cured cultures. This culture had therefore been cured of two of its prophages. Colony-centered plaques (phage $1C^{tox+}$) and turbid plaques (phage $2C^{tox-}$) were produced on bacterial lawns of strain AO28 by phages isolated from cell-free lysates of the parent strain 468C. These phages were purified by five successive single-plaque isolations on strain AO28.

Table 1. Relation of Phages of Type C Strain 468C to Toxigenicity and Sensitivity of Strain AO28

Bacterial strain and phage	Toxigenicity ^a	Sensitivity to phage	
		1C	2C
AO28	-	+	+
AO28 (1C)	+	-	+
AO28 (2C)	-	+	-
AO28 (1C, 2C)	+	-	-

^aProduction of predominant C_1 and minor D toxin

The relationship of each of these phages to the toxigenicity of strain AO28 was studied using procedures previously described (4). Table 1 summarizes the results of these experiments. When strain AO28 was infected with phage 1C, it concomitantly produced dominant C₁ and minor D toxins and displayed immunity to infection by the homologous phage. Phage 2C, however, did not induce strain AO28 to produce C₁ and D toxins, but the infected cultures, and all other cultures irrespective of phage involvement, did continue to produce C₂ toxin.

To determine whether the continued participation of phage 1C was necessary to maintain toxigenicity, strain AO28 (1C) was cultured in TYG medium containing antiserum against phage 1C and plated on TYG agar. Isolates that were resistant to phage 1C continued to carry phage 1C and to produce C₁ and D toxins. On the other hand, isolates cured of phage 1C simultaneously ceased to produce C₁ and D toxins. These "nontoxigenic" isolates, however, resumed the production of C₁ and D toxins after they were reinfected with phage 1C. This curing and reinfection cycle was repeated with strain AO28 and other "nontoxigenic" isolates from type C strain 468C and in every case the production of the C₁ and D toxins depended upon the continued participation of phage 1C. These results therefore emphasize the necessity of specific phages in the production of C₁ and D toxins by *C. botulinum* type C.

Strains of type C isolated from the different areas of the world were also examined to determine whether their phages also governed toxigenicity. In these experiments, spores from different strains were heated to 70°C for 15 minutes to inactivate free phage and plated on TYG agar. Vegetative cells were grown in TYG broth containing acridine orange or treated with ultraviolet irradiation and survivors plated on TYG agar. After anaerobic incubation, isolates were tested for phage-sensitivity and toxin production. All three methods yielded phage-sensitive derivatives which had simultaneously lost their ability to produce C₁ and D toxins. Strain 164 lost its phage and toxigenic characteristic during passage in EM medium (Table 2). Each of the "nontoxigenic" derivatives except the isolates from strain 6816 could be converted back to the toxigenic state (again produced C₁ and D toxins) when they were reinfected with specific TOX⁺ bacteriophages from the toxigenic parent cultures. These converted cultures continued to produce TOX⁺ phages and C₁ and D toxins during subculture in TYG or egg meat medium (EM). They also responded like type C strain 468C in being immune to the infection by the TOX⁺ phages of the parent strain as long as they remained toxigenic and carried the corresponding TOX⁺ phage.

With the exception of strain 162 or its phage-sensitive derivatives, all strains of type C produced the C₂ toxin

Table 2. Relation of bacteriophages to the toxigenicity of different strains of *C. botulinum* type C

Strain number	Method of obtaining cured cultures	Number of cultures			
		Tested	"Nontoxic"	Producing C ₂ toxin	Converted to toxigenicity by phage
6816	Acridine orange	80	3	+	-
165	Spores	80	2	+	+
153	Spores	58	8	+	+
162	Acridine orange	64	2	-	+
162	Spores	40	1	-	+
3296	Spores	79	9	+	+
571	Spores	73	7	+	+
C ₃	Acridine orange	92	9	+	+
C ₈	Acridine orange	78	20	+	+
203	Acridine orange	89	5	+	+
2337	Acridine orange	102	40	+	+
6513	Acridine orange	160	86	+	+
SKM	Acridine orange	105	5	+	+
468C	Acridine orange	68	2	+	+
468C	Ultraviolet	106	15	+	+
460	Acridine orange	63	25	+	+
164	Passage in media	---	--	+	+

before and after they were cured of their TOX⁺ prophages (Table 2). These results confirmed the earlier findings that the production of C₂ toxin was not governed by any of the bacteriophages used in these studies.

When the "nontoxigenic" isolates were tested for their sensitivity to the purified phages of the different type C cultures, five of the isolates were sensitive to numerous TOX⁺ phages produced by toxigenic type C strains (Table 3). The remaining ten derivatives were sensitive only to the phages of the toxigenic parent culture. Each of the TOX⁺ phages converted the "nontoxigenic" strains to the toxigenic state. Similar results have been reported with other type C strains (12,13,20-23). These results indicate that specific TOX⁺ phages play a common role in the toxigenicity of different strains of type C.

Toxigenicity of Type D strains

The same procedures used to determine the involvement of bacteriophages in the toxigenicity of type C strains were also employed to study the toxigenicity of type D strains 1873 and South African. Strain 1873 produced the dominant D toxins and minor toxins C₁ and C₂. The South African strain, however, produced only the dominant D toxin.

Table 3. Host Range of Bacteriophages Isolated from *C. botulinum* Type C Strains

"Nontoxigenic" host	Number of type C strains		
	Tested	Produced phage that infected "nontoxigenic" host	Converted "nontoxigenics" to toxigenic state
AO50	21	13	13
AO28	21	12	12
HS46	21	10	10
HS31	21	8	8
HS34	21	12	12

When the South African strain of type D was studied, a greater number of "nontoxigenic" isolates were obtained from sporulated cultures than from vegetative cells cultures in TYG medium containing acridine orange. All of the "nontoxigenic" isolates from both sources were sensitive to phage 1D^{tox+} from the toxigenic parent culture. This phage converted each isolate to produce the dominant type D toxin. Toxigenic isolates continued to carry and to be immune to phage 1D.

Further studies were made with "nontoxigenic" isolate AO20. This isolate maintained its "nontoxigenic" state and sensitivity to phage 1D during numerous passages in EM medium over a 5-year period. It also maintained the toxigenic characteristic as long as it was infected with phage 1D. Strain AO20 (1D) was permitted to sporulate and the spores were washed, centrifuged, and plated on TYG agar. Following anaerobic incubation, colonies were again tested for their toxigenicity and phage-sensitivity. Of the 39 isolates selected, 19 were "nontoxigenic" and sensitive to phage 1D. After infection with phage 1D, each of the 19 isolates were converted to the toxigenic state and continued to produce type D toxin as long as they carried phage 1D. Occasionally, a toxigenic culture would become "nontoxigenic" during passage in EM medium. These "nontoxigenic" cultures were invariably sensitive to phage 1D and could be converted to the toxigenic state merely by phage infection.

These studies were also extended to type D strain 1873 to determine whether phages were involved in the toxigenicity of other type D strains that produce not only dominant D toxin but also minor C₁ and C₂ toxins. This toxigenic culture carried two phages designated as phage 2D^{tox+} and 3D^{tox-}. Of 214 isolates examined from strain 1873 following acridine orange treatment, 23 were "nontoxigenic" and sensitive to phage 2D^{tox+}. One of these isolates, AO113, was also sensitive to phage 3D^{tox-}. Phage 2D converted each of the "nontoxigenic" isolates to the toxigenic state and dominant D and minor C₁ toxins were again produced. Phage 3D, however, did not participate in the production of any of these toxins. All of the "nontoxigenic" and toxigenic isolates from strain 1873 continued to produce C₂ toxin which required trypsin activation to demonstrate toxicity.

Strain 1873 resembled the South African strain in that subcultures would occasionally lose their ability to produce D and C₁ toxins. These "nontoxigenic" cultures were always sensitive to phage 2D and could be converted to the toxigenic state by phage 2D.

Production of C₂ Toxin by Type C and D Cultures

Of the 21 different type C cultures isolated from six different countries, all except one produced C₂ toxin. This strain was isolated in England. The C₂ toxin from 15 of the strains cultured in EM medium required trypsin activation before toxin could be detected.

Type C cultures that had lost their toxigenic properties during transfer in laboratory media were received from other research laboratories labeled as "nontoxigenic" strains. Even though these strains did not produce C₁ and D toxins, 8 of the 15 strains did produce C₂ toxin which was detectable only after trypsin activation.

Recent studies indicate that the production of the C₂ toxin is correlated with the sporulation of type C cultures (18). The larger the sporulation, the higher the titer of C₂ toxin. When the spore populations were less than 10⁴/ml of culture, C₂ toxin was not detectable in the culture supernatant fluids.

The optimum pH for trypsin activation of toxins from non-proteolytic strains of *C. botulinum* types B, E, and F is 6.0. When C₂ toxin was studied, the highest titers were obtained following trypsin activation at pH 6.5 (5).

Strain 1873 was the only type D culture that produced C₂ toxin. This toxin required trypsin treatment to demonstrate toxicity and was neutralized by antiserum prepared against the toxin of type C strain 468C. The C₂ toxins from type C and D strains therefore appear to be antigenically closely related (5).

INTERCONVERSION OF *C. BOTULINUM* TYPE C AND D STRAINS BY BACTERIOPHAGES

Strain 1873 was identified as *C. botulinum* type D because it produced the dominant D toxin. When strain 1873 was cured of phage 2D^{tox+}, it could no longer be classified as type D because of its inability to produce D toxin. These "nontoxigenic" phage-sensitive derivatives, however, continued to produce C₂ toxin and became indistinguishable from "nontoxigenic" type C cultures.

The similarities in the characteristics of these cured derivatives of type C and D strains suggested that type C and D strains might arise from a common culture infected with different phages. To test this hypothesis, strain AOAll3 was tested for its sensitivity to the phages of different type C strains. Phage 4C^{tox+} from type C strain 153 infected AOAll3

and converted it to the toxigenic state in which C_1 toxin was dominant. When the cured derivatives of type C were tested for their sensitivity to phage $2D^{tox+}$ from 1873, only derivative HS15 from type C strain 153 was sensitive. Phage 2D converted HS15 to the toxigenic state and D toxin was dominant. As a result, derivatives HS15 and AO113 became common hosts for both type D phage 2D and type C phage 4C. These cultures could therefore be converted to type D or to a type C merely by exchanging the TOX^+ phage (Table 5). Cultures infected with phage 4C were immune to infection by phage 2D and vice versa. Each culture irrespective of phage involvement produced C_2 toxin that required trypsin treatment to demonstrate toxicity.

Cultures AO113(4C), AO113(2D), HS15(4C), and HS15(2D) were permitted to sporulate and "nontoxigenic" derivatives were again isolated. These derivatives each became sensitive to phages 4C and 2D and when infected they again produced the dominant C_1 or D toxins, respectively.

These curing and reinfecting experiments were repeated three times and in each instance the production of toxin and the toxin type depended upon the continued presence of specific TOX^+ phages.

Interconversion of types C and D by bacteriophages was also observed in another group of strains that did not produce C_2 toxin. Strain HS37 (derived from type C strain 162) was not only sensitive to phage $3C^{tox+}$ of the parent strain but also to phage $1D^{tox+}$ from the South African strain of type D. Table 6 summarizes the results of the relationship of phage 1D and 3C to the type of toxin produced by strain

Table 5. Relation of Bacteriophages 2D and 4C to the Toxigenicity of Bacterial Strains AO113 and HS15

"Nontoxigenic" cured cultures	Phage	Number of cultures		Toxin neutralized by antiserum
		Toxigenic and phage producers	Tested	
AO113	2D	20	20	Type D
AO113	4C	37	37	Type C
HS15	2D	20	20	Type D
HS15	4C	20	20	Type C

Table 6. Relation of Bacteriophages 1D and 3C to the Toxigenicity of Strain HS37

Infecting phage	Number of cultures		Toxin neutralized by antiserum
	Tested	Converted to toxigenic state	
1D	40	30	Type D
3C	40	40	Type C

HS37. Infection of HS37 with phage 3C resulted in the production of dominant C_1 toxin whereas infection with phage 1D resulted in the production of the dominant D toxin. Of 40 TYG cultures arising from plaque material from phage 1D, only 30 were toxigenic. The ten "nontoxigenic" isolates were retested and found to be phage-sensitive and capable of producing D toxin when they were infected with phage 1D.

Strain HS37(1D) produced only 10 MLD of D toxin per ml. When the culture supernatant fluid was treated with trypsin, the toxicity increased to 2000 MLD/ml. In contrast, the South African type D strain which also carried phage 1D produced 10,000 MLD/ml of type D toxin and the titer was increased only 10-fold by trypsin treatment. This difference in the toxicity suggests a difference in the enzymes produced by the two cultures.

Strain HS37 (1D) often lost its phage and reverted to the "nontoxigenic" state after three or four transfers in TYG or EM medium. The production of the D toxin could be restored by merely reinfected the "nontoxigenic" isolates with phage 1D. The maintenance of phage 1D and toxigenicity by strain HS37 could be continued for longer periods of time when the EM medium contained 2% sodium chloride.

INTERSPECIES CONVERSION OF *CLOSTRIDIUM BOTULINUM* TYPE C TO *CLOSTRIDIUM NOVYI* TYPE A BY BACTERIOPHAGES

C. botulinum and *C. novyi* are pathogenic anaerobes that are characterized by their ability to produce powerful toxins.

The *C. botulinum* group produce neuromuscular toxins that are responsible for botulism in man and animals. *C. novyi* also produce lethal toxins and are often found in gas gangrene infections of man and in other diseases of animals.

The species *C. novyi* includes a heterogeneous group of organisms that is divided into types A, B, C, and D on the basis of different toxins produced. The production of lethal alpha toxin is the characteristic that unites types A and B. When types A and B strains were cured of their TOX⁺ phages, they discontinued the production of the alpha toxin. As a result, "nontoxigenic" type A cultures no longer resembled the other *C. novyi* types, but instead became closely related to "nontoxigenic" *C. botulinum* type C and D strains (8,9). In comparison, when the *C. novyi* type B strains lost their TOX⁺ phages and ceased to produce alpha toxin, they closely resembled *C. novyi* type D (*C. haemolyticum*) in that they continued to produce the same lethal beta toxin and other minor antigens. The main characteristic in the identification and differentiation of *C. botulinum* types C and D and *C. novyi* type A therefore is the toxins produced.

To determine the relationship of these two clostridial species, the phage-sensitive "nontoxigenic" derivatives of types C and D were tested for their sensitivity to the phages of 8 different strains of *C. novyi* type A. Strain HS37 (from type C strain 162) was found to be sensitive to the phages of *C. novyi* type A strain 5771. Cell-free lysates of strain 5771 contained two different phages. When phage NA1^{tox+} infected strain HS37, the culture concomitantly produced the lethal alpha toxin of *C. novyi*. Phage NA2^{tox-} also infected strain HS37, but showed no relationship to any of the toxins produced.

In earlier sections of this paper, strain HS37 was reported to be sensitive to type D phage 1D and type C phage 3C. The relationship of the phages NA1, 1D, and 3C to the toxigenicity of strain HS37 therefore was studied. When type C strain 162 was cured of phage 3C, it became "nontoxigenic" and a common host to phages NA1, 1D, and 3C (Table 7). Infection of strain HS37 with phage NA1 converted it to *C. novyi* type A and dominant alpha toxin was produced. If this culture was cured of phage NA1 and infected with phage 3C, then it was converted to *C. botulinum* type C, and the C₁ toxin was dominant. Phage-sensitive derivatives isolated from type C culture HS37 (3C) could then be infected with phage 1D and the culture was identified as type D because of the dominant D toxin. A phage-sensitive strain of clostridia therefore could be converted to *C. botulinum* type C or type D or to *C. novyi* type A by merely exchanging the bacteriophages. These

Table 7. Effect of Different Phage on Toxigenicity of Strain HS37

Phage	Number of cultures			Neutralized by antiserum of:
	Tested	Toxic	Produce phage	
3C ^{tox+}	40	40	40	<i>C. botulinum</i> type C
1D ^{tox+}	40	40	40	<i>C. botulinum</i> type D
NA1 ^{tox+}	40	40	40	<i>C. novyi</i> type A
NA2 ^{tox-}	40	0	40	-----

studies show that the toxigenicity of *C. botulinum* types C and D and *C. novyi* types A and B depends upon the continued participation of specific TOX⁺ phages.

STABILITY OF PHAGE HOST RELATIONSHIP

The high frequency of isolating "nontoxigenic" phage-sensitive derivatives from toxigenic strains of *C. botulinum* types C and D and *C. novyi* types A and B following acridine orange and ultraviolet irradiation treatments indicated that the phage-host relationship was unstable. Further evidence of this instability was obtained when isolates from toxigenic sporulated cultures were tested for their phage immunity and toxigenicity (Table 8). Even though the degree of instability varied markedly from strain to strain, all of the toxigenic strains yielded isolates that had lost their phages and immunity. These results suggested that a pseudolysogenic relationship existed between the phage and host.

In order to confirm these findings, toxigenic strains were transferred twice a day in TYG medium containing antiserum against the specific phages. Examples of the results are summarized in Table 9. A very high percentage of the isolates tested were phage-sensitive, and this percentage increased as the number of passages in phage antiserum increased. These results imply that the bacterial cells lose their phages during culture but are protected from reinfection by phage

Table 8. Loss of Phage and Toxigenicity through Spore State of *C. botulinum* Type C and *C. novyi* Type A

Strain	Number of colonies	
	Tested	"Nontoxigenic" and phage-sensitive
3296 ^a	79	9
571	73	7
165	80	2
460	23	12
J C	97	9
162	40	4
SKM	30	6
C ₃	40	4
C ₈	20	20
203	89	5
X-200	40	39
2337	40	40
6513	86	16
468C	37	10
201	2	2
SA ^b	39	19
5771 ^c	50	25

^a*C. botulinum* type C strain

^b*C. botulinum* type D

^c*C. novyi* type A

antiserum. In the absence of antiserum, toxigenic cultures also lose their phages, but they can be reinfected by free phages which are present in an actively growing culture. These different results therefore support the fact that a pseudolysogenic relationship exists between phages and their host in *C. botulinum* types C and D and *C. novyi* type A and B strains.

Results from these studies demonstrate the important role that specific phages play in the production of *C. botulinum* C₁ and D toxins and the alpha toxin of *C. novyi* types A and B. Because of the pseudolysogenic relationship between the host and phage, these cultures occasionally lose their phages in nature and become "nontoxigenic." Depending on the presence

Table 9. Effect of Cultivation of Toxigenic Cultures in TOX⁺ phage antiserum on phage-sensitivity and toxigenicity

Strain	Number of transfers in antiserum	Number of cultures	
		Tested	Phage-sensitive and "nontoxigenic"
SKM ^a	3	40	6
	7	40	15
468C ^a	7	39	27
S.A. ^b	7	37	29
8024 ^c	6	194	19

^a*C. botulinum* type C

^b*C. botulinum* type D

^c*C. novyi* type B

of other phages, these "nontoxigenic" strains could be induced to produce *C. botulinum* toxins C₁ or D or the alpha toxin of *C. novyi*. Because of this role, these bacteriophages are very important to the identification of the pathogenic clostridia and also to the corresponding disease that they cause.

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